**Microbiota predict *Clostridium difficile* severity in germ-free mice colonized with human feces**

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**Abstract**

*Clostridium difficile* causes diarrheal disease when it successfully colonizes a dysbiotic gut microbial community. Current mouse models to study *C. difficile* infection (CDI) rely on pre-treatment with antibiotics to disrupt the mouse microbiome prior to *C. difficile* inoculation. This is an effective model for many studies but does not allow for analysis of human-associated microbial community members that support or prevent *C. difficile* colonization and expansion. To study human-associated microbes in the context of CDI, we colonized germ-free C57BL/6 mice with one of 16 human fecal samples from diarrheal or healthy donors and challenged with *C. difficile* 14 days later. Five unique donor-mice combinations resulted in severe CDI while the remaining 11 only experienced mild disease. Both healthy and diarrheal donors were susceptible to colonization and severe symptoms of CDI. To determine if specific microbes were associated with disease severity outcomes, we built a classification Random Forest machine learning model based on relative abundance data of the communities on day zero. The model identified a number of bacterial populations associated with the development of severe CDI, including *Bacilliales*, *Ruminococcaceae*, *Ruminococcus, Staphylococcus, Streptococcus* and *Bacteriodetes.* Additionally, a regression model accurately predicted colonization levels of *C. difficile* at one to ten days post-infection. This model explained 99% of the variance in the number of CFU isolated from mouse stool. Members of *Lachnospiraceae, Parabacteroi- des, Bacteroidales, Bacteroidetes, Porphyromonadaceae* and unclassified *Bacteria* were predictive of future *C. difficile* colonization levels. Finally, challenging these mice with different strains of *C. difficile* revealed that susceptible human-associated microbial communities were prone to severe disease independent of strain. Taken together these results suggest that human-associated microbial communities can be recapitulated in germ-free mice and used to characterize dynamics of CDI. Because both healthy and diarrheal patients were susceptible to severe CDI, machine-learning models are useful to identify bacterial populations that allow colonization and contribute to the development of *C. difficile* associated disease in humans.

**(Importance?)**

**Introduction**

*Clostridium difficile* is an opportunistic pathogen of the human lower gastrointestinal tract. Disruption of the native microbial community of the gut by antibiotics is the most common risk factor for development of *C. difficile* infection (CDI) (Britton and Young 2012). *C. difficile* is a spore-forming bacteria and can persist on abiotic surfaces and is not readily killed by ethanol-based hand-sanitizers, putting hospital patients particularly at risk. Indeed, ~12% of hospital acquired infections in the United States are due to *C. difficile* and is responsible for up to 15,000 deaths annually (Lessa et al 2015).

Murine models to study CDI typically rely on treating conventionally raised mice with antibiotics either in drinking water or by injection to induce susceptibility (cite). These provide a convenient way to study *C. difficile* pathogenesis and virulence factors. Numerous microbiome studies have been performed to determine the antibiotic classes (alyx), starting microbial community (cite) and metabolites (casey?) that impact development and severity of CDI. While informative, these studies are somewhat removed from human disease because they only examine mouse-associated microbial communities.

Gnotobiotic or germ-free mouse models have been used for a range of studies of CDI, including assessment of species-specific interactions between *C. difficile* and competing microbial community members, analysis of nutrient restriction, *in vivo* transcriptomics of *C. difficile* and examination of host immune response to CDI (Ng et al 2013, Pawlowski 2010, Reeves 2012, ). Further, CDI therapeutics such as antibiotics and fecal microbiota transplants have been tested extensively in a gnotobiotic-piglet or piglet-to-gnotobiotic-mouse model of disease (steel 2013, kim 2014, Diao 2016). Pigs have a longer digestive tract with components more similar to humans than mice and are typically infected by strains typical in human infection (cite). However, the microbes that survive in mice and pigs are typically not similar to those of the human gut (cite).

The power of the gnotobiotic models to study CDI has been further realized by first inoculating germ-free mice and piglets with human stool microbes. In one study, germ-free piglets were acutely colonized with human feces for one week and then treated with tigecycline. After challenge with *C. difficile* none of the antibiotic-treated piglets succumbed to infection, while some of the untreated human-colonized pigs did(Kim et al 2014). Further, germ-free mice colonized with human feces were bred over several generations to create a cohort of mice with similar human-derived microbiomes (Collins 2015). These mice were subsequently treated with a five-antibiotic cocktail to induce dysbiosis and then were successfully colonized by *C. difficile* (Collins 2015). While informative, these studies were limited in their use of only one human donor as input inoculum.

To test the diversity of human microbiomes and their susceptibility to CDI, here we colonized germ-free mice with 16 different human stool donors. For the first time, we characterized human-associated microbiome response to *C. difficile* challenge. Additionally, the use of machine-learning models allowed us to build a predictive model that classified “at-risk” microbiomes prior to infection with *C. difficile*. These findings show that human-associated microbiomes can be at risk for CDI even in the absence of antibiotics and that study of mice colonized with human feces provides a range of clinical outcomes. (this last line sucks)/

**Results**

1. Germ-free mice inoculated with human feces as model for *C. difficile* infection
2. *C. difficile* infection dynamics
3. Random Forest predicts CDI severity from day 0
4. Microbial community on day 0 predicts future *C. difficile* CFU
5. Propensity for severe CDI is community-dependent and strain-independent
6. histology

**Discussion**

* Discuss results, caveats about mouse weights and differneces between donors
* No donors were colonization resistant, discuss donor differences
* Discuss prediction methods and outcomes
* Discuss potential mechanisms for interesting OTUs
* Discuss different strain results
* Future work blah blah

**Methods**

* Mice ULAM number
* Donor stool ERIN IRB shit
* Bacteria/plating
* Sequencing
* Data analysis
* Machine learning

**Acknowledgments**

Lab, sequencing core, Jhansi

**Citations**

**Figure 1: Germ-free mice inoculated with human feces as a model for *C. difficile* infection**

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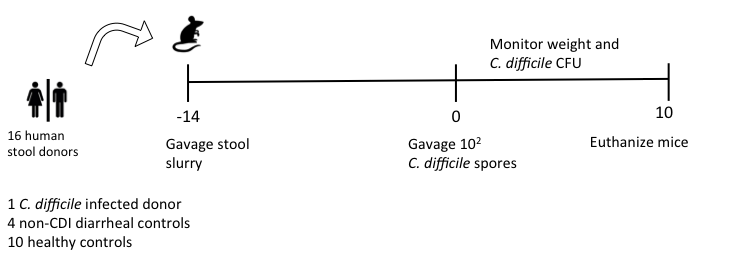


Figure 1. Germ-free mice inoculated with human feces as a model for *C. difficile* infection. A) Stool was collected from 16 healthy, diarrheal and CDI patients and inoculated into 3-4 germ-free mice per donor by oral gavage. After allowing the community to stabilize for 14 days, mice were orally gavaged with 100 spores of *C. difficile* strain 431. Weight and stool CFU was monitored for up to 10 days post infection.

Need new NDMS by clinical outcome here

**Figure 2. *C. difficile* infection dynamics**

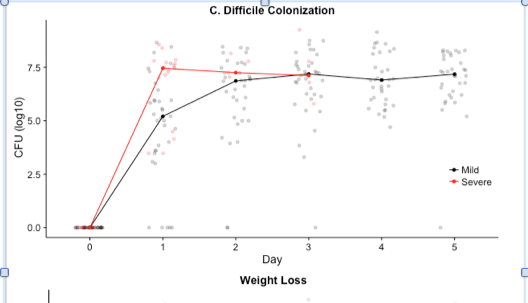
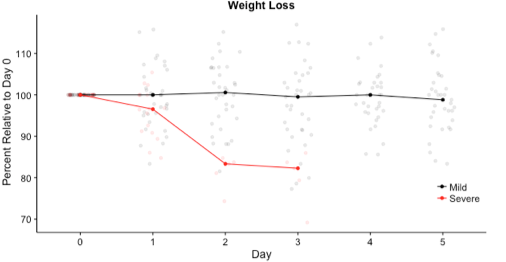
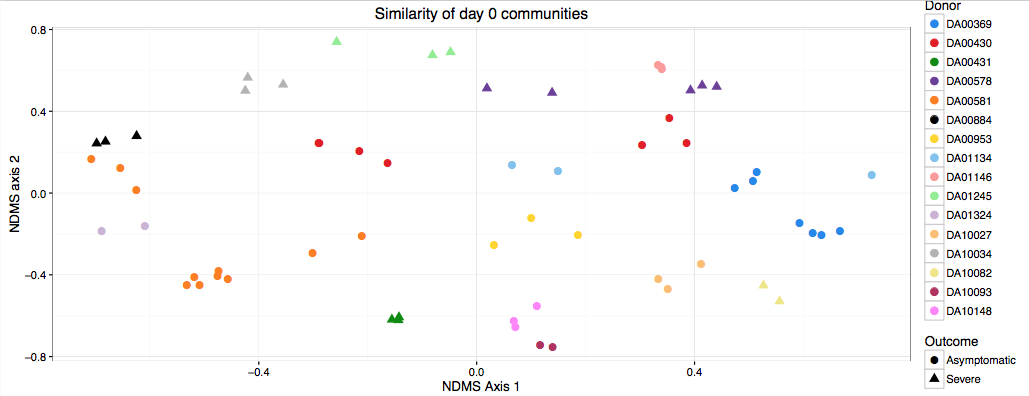
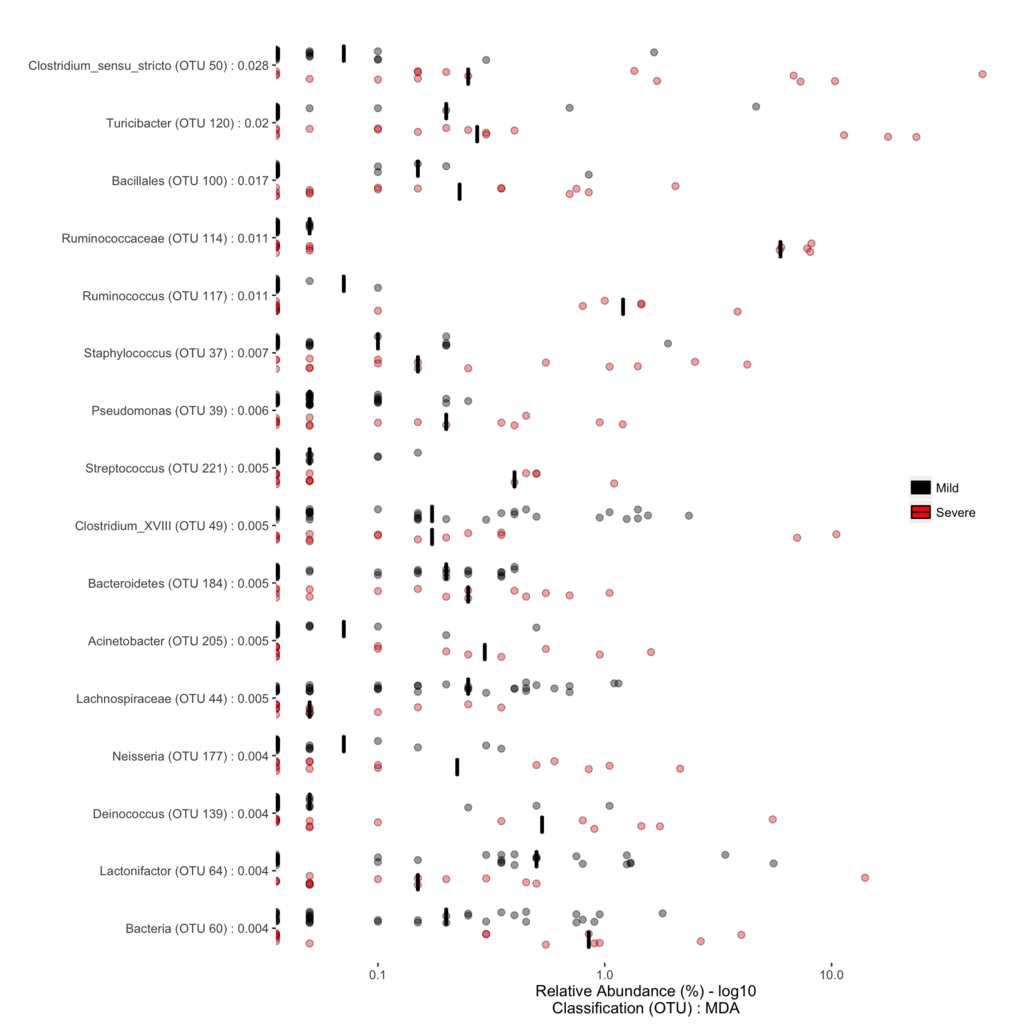
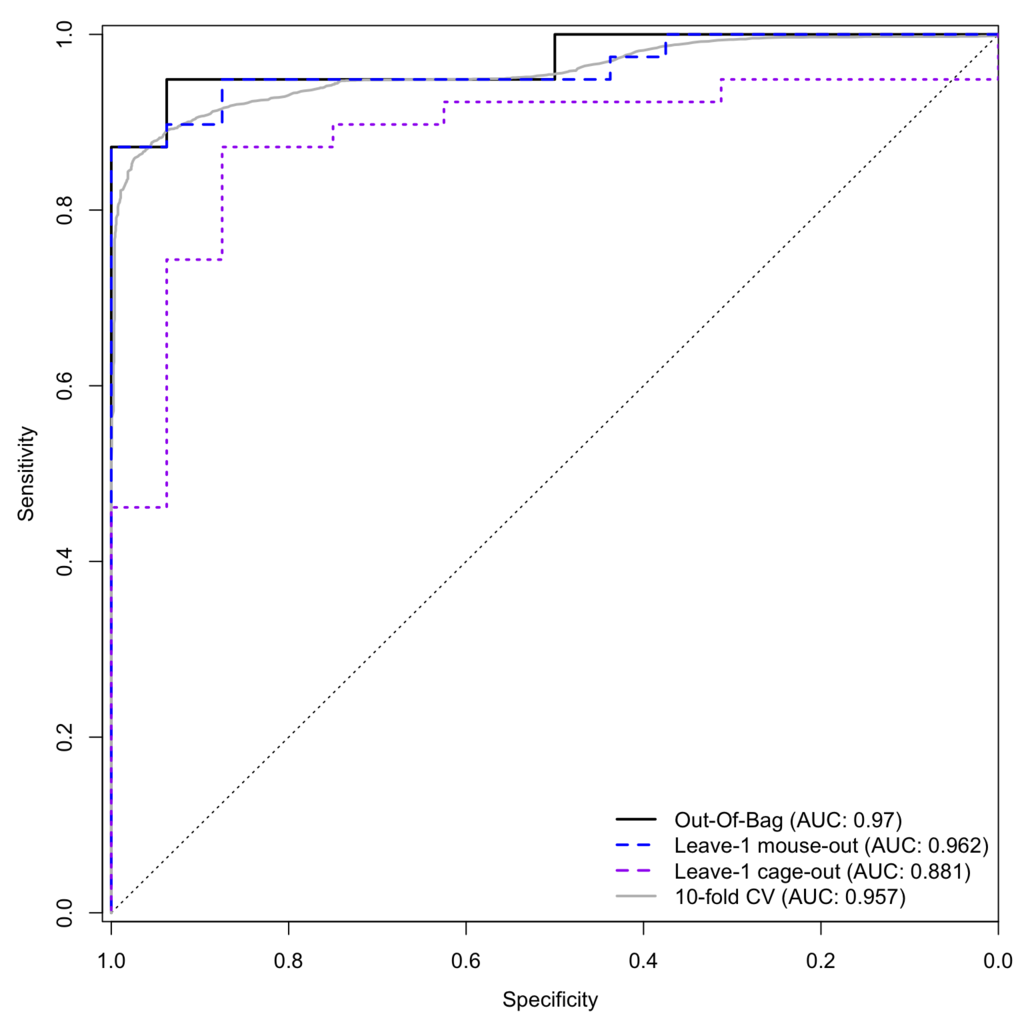
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Figure 2. *C. difficile* infection dynamics. A) *C. difficile* CFU was enumerated by plating of mouse stool pellets daily. Each point represents a mouse and the lines represent the mean of mice in each cage and error bars are interquartile ranges. Red lines and points correspond to mice that succumbed to severe disease, black lines and points correspond to mice that had mild or no disease. B) Mouse weights were recorded daily percent weight loss calculated for each mouse. Data presented as the mean of mice in each cage. Mice that succumbed to severe infection typically lost a significant amount of weight by day 1 or 2 post infection. Red lines correspond to severely ill mice, black to mice with mild disease.

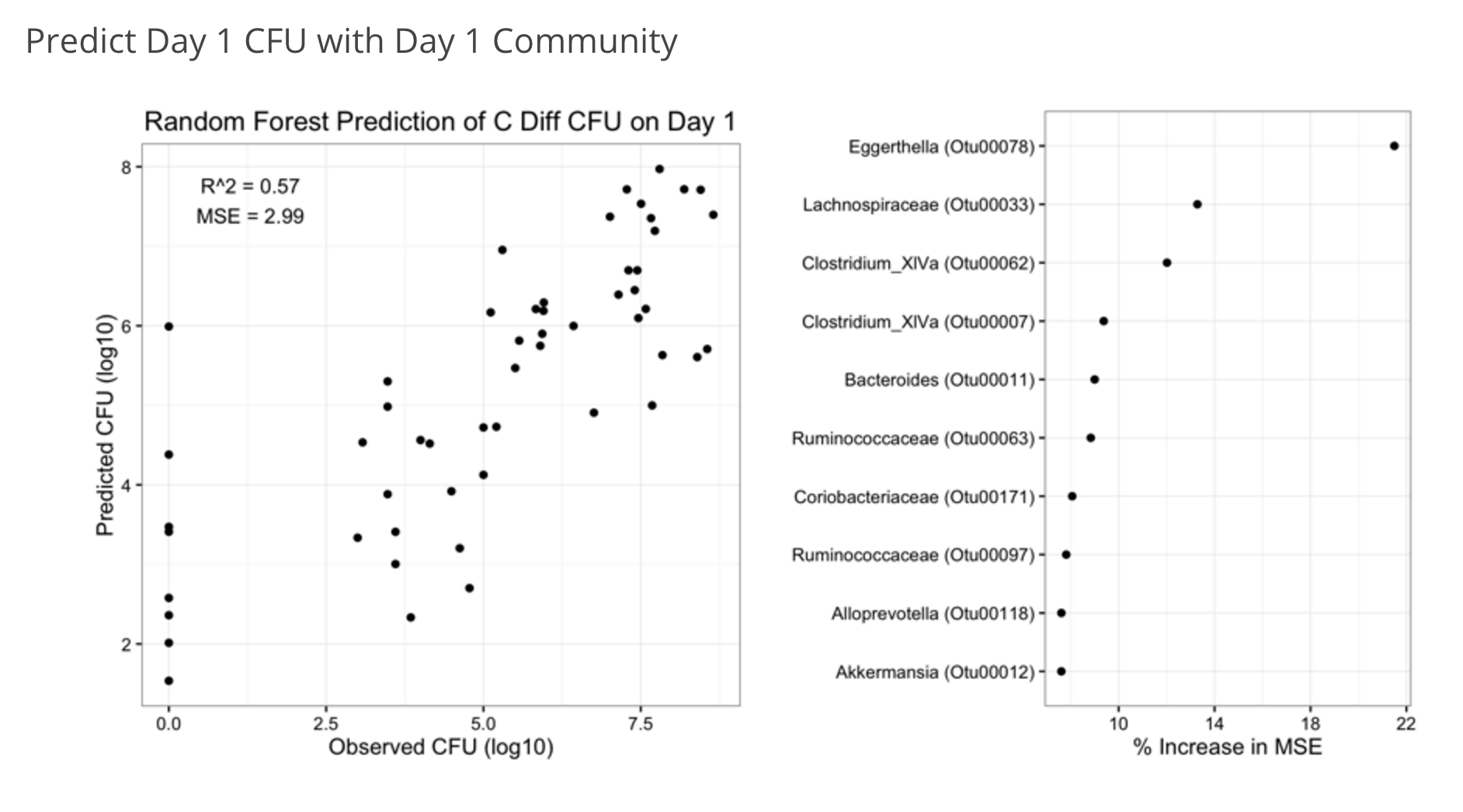


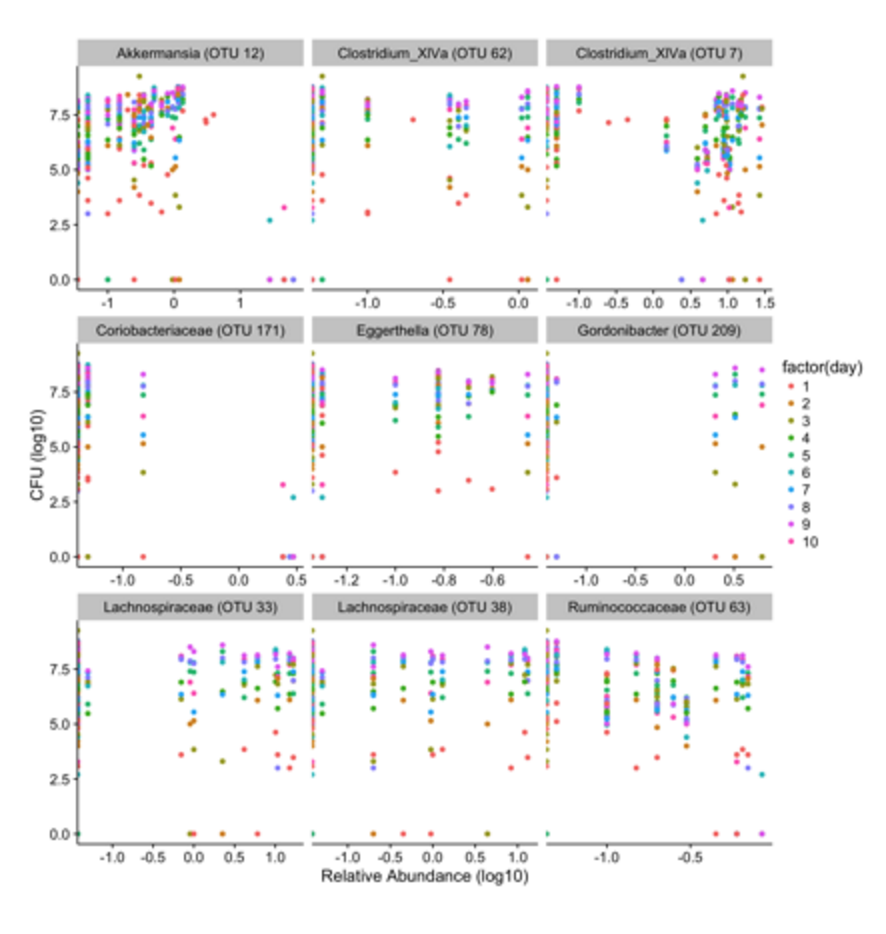
B) NDMS ordination of the stool communities on day 0. Each symbol represents one mouse and is colored by donor. Circles represent mice that survived the 10 days of infection and triangles represent those who suffered severe disease.

**Figure 3. Random forest predicts CDI severity from day 0 microbiome.**

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**Figure 4. Microbial community on day 0 predicts future *C. difficile* CFU**

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**Figure 5. Propensity for severe CDI is community-dependent and strain-independent**

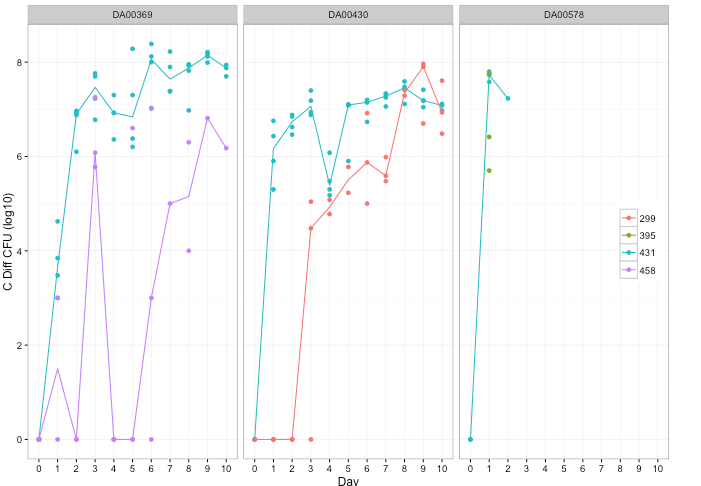
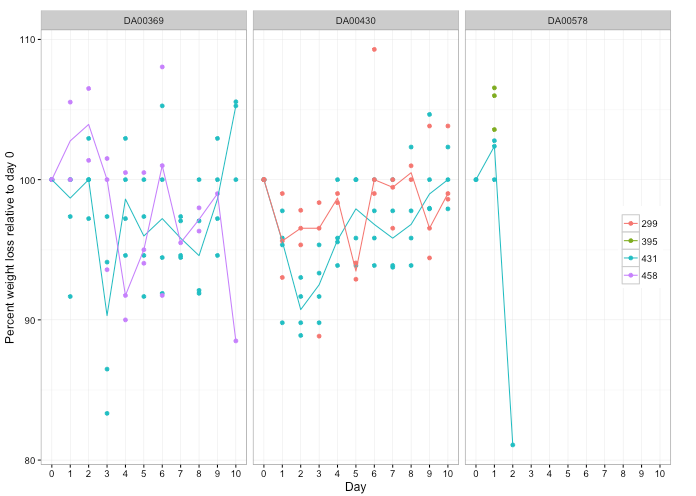
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Figure 5. Infection of mice with different *C. difficile* strains. 3 strains of *C. difficile* were used to infect mice colonized with susceptible (DA00578) or resistant (DA00369, DA00430) human donor stool. A) *C. difficile* stool CFU was enumerated over 10 days. B) Percent weight loss was calculated each day for each mouse. In both plots, each mouse is a point and lines represent the mean of each cage.